The role of a small RNA in Biofilm Formation

A Senior Honors Thesis

Presented in Partial Fulfillment of the Requirements for graduation *with research distinction* in Microbiology in the undergraduate colleges of The Ohio State University

by

Kayla Mae Humenansky

The Ohio State University

December 2010

Project Advisor: Dr. Michael Ibba, Department of Microbiology

Abstract

An iron-regulated small RNA (sRNA), $sRNA^{other}$, was recently identified in *Bacillus cereus* ATCC 14579. Many iron-regulated sRNAs are involved in maintaining iron homeostasis. In addition, some of these sRNAs are also involved in biofilm formation. The deletion of $sRNA^{other}$, from the *B. cereus* ATCC 14579 genome leads to changes in cell wall morphology and antibiotic resistance (10). These findings led to our investigation of biofilm formation of *B. cereus* ATCC 14579 and *B. cereus* ΔsRNA^{other}. Biofilm formation was measured both qualitatively and quantitatively at the liquid-air interface. Qualitative results showed differences in biofilm structure between wild-type *B. cereus* and *B. cereus* ΔsRNA^{other} in media containing the iron chelator 2',2'-dipyridal (dip). Whereas quantitative results showed that *B. cereus* ATCC 14579 had significantly greater biofilm formation than the *B. cereus* ΔsRNA^{other}, there was no significant difference in biofilm formation between the cultures containing dip and those without. Therefore, indicating that $sRNA^{other}$ plays a role in biofilm formation.

Introduction

Transfer RNAs (tRNAs) are essential for translation of the genetic code. Past investigations have shown tRNAs ability to regulate gene expression by acting as sensors of the translation status of the cell. It has also been hypothesized that these exceptionally stable molecules could provide a structural framework for small RNAs (sRNAs). (10) Recent studies have demonstrated that a pseudo-tRNA from *Bacillus cereus*, $sRNA^{other}$, does not associate with polysomes; therefore suggesting a role outside of translation (9, 13). Deletion of $sRNA^{other}$ results in changes in cell wall morphology and notably reduces

resistance to various unrelated antibiotics including vancomyin, puromycin, novobiocin, and rifapentin.

Further investigations demonstrated the significance of extracellular iron levels in the expression of $sRNA^{other}$. Expression of $sRNA^{other}$ can be altered by changes in extracellular iron concentration. The presence of three putative ferric uptake regulator (Fur) binding sites in the putative promoter region of the corresponding gene indicated this relationship.

Further investigations of $sRNA^{other}$ were conducted to gain a better understanding of its functional sequence and size. Mapping of $sRNA^{other}$ revealed a much larger RNA than originally hypothesized. Possible variations due to transcription from different predicted promoters and potential processing sites were also uncovered (10). Overall, these findings indicate that $sRNA^{other}$ is an iron-regulated sRNA important in antibiotic resistance. (10) Other iron-regulated sRNAs have been shown to contribute to virulence. In a recent study, biofilm formation was found to be affected by an iron-regulated sRNA, RhyB, in *Vibrio cholerae* (11). These findings led to our investigations concerning the potential role of $sRNA^{other}$ in biofilm formation in *Bacillus cereus*.

Bacillus cereus is a gram-positive, spore-forming, facultative anaerobe commonly identified as the causative agent of food-borne illnesses (1). It is a ubiquitous organism that can easily contaminate food production or processing systems and water treatment pipes. Contamination may result from its ability to form biofilms that are highly resistant to cleaning procedures (1, 2).

Biofilms consist of structured, surface-attached bacterial communities encased in a secreted polymeric matrix (3), which can form on virtually any surface once it has been

conditioned by proteins and other molecules present in the environment (4). Biofilm formation results from microbes reversibly attaching to a conditioned surface and releasing polysaccharides and proteins, which allow the microbes to adhere more stably to the surface. Biofilms thicken and mature as the microbes reproduce and secret additional polysaccharides and proteins resulting in a complex, dynamic community of microorganisms. While some biofilms constitute only one species, biofilms more commonly consist of a variety of microbial species living together (4).

Biofilms are beneficial to microbes because they provide protection from harmful agents such as ultra violet light, antibiotics, and other antimicrobial agents. The resistance of biofilms to many antimicrobial agents can cause serious health issues (4). Another health concern with biofilms is their ability to disperse, releasing cells into the environment to resume planktonic growth (3, 4). Planktonic cells can then move throughout the environment and colonize new surfaces.

Antibiotic resistance mechanisms in biofilms are not fully understood; however, some mechanisms of antibiotic resistance, such as modifying enzymes, target mutations and efflux pumps, do not appear to be responsible for the protection of the bacteria colonized within a biofilm (5). It has been hypothesized that biofilm formation can profoundly reduce the susceptibility of antibiotic sensitive bacteria, which have no known genetic basis for antibiotic resistance. Biofilms may reduce susceptibility of an antibiotic by causing slow or incomplete penetration of the antibiotic or initiating the development of an altered chemical microenvironment within the biofilm (5).

Slow penetration can lead to deactivation or absorption of the antibiotic within the biofilm. For example, biofilm formation by *Klebsiella pneumoniae* aids in ampicillin

resistance by deactivating the antibiotic in the surface layers of the biofilm (6). Deactivation occurs more rapidly than diffusion and the antibiotic becomes ineffective by the time it penetrates the extracellular matrix of the biofilm. Alteration of the chemical microenvironment within the biofilm includes local accumulation of waste products which can alter the pH within the biofilm, once again leading to inactivation of certain antibiotics (5).

In this study, the role of $sRNA^{other}$ in biofilm formation in *B. cereus* was investigated. Biofilm formation was qualitatively and quantitatively assessed using microscopy and absorbance measurements, respectively. WT *B. cereus* ATCC 14579 was shown to form greater and more complex biofilms than a $sRNA^{other}$ deletion strain, therefore indicating a role for $sRNA^{other}$ in biofilm formation.

Materials and Methods

Qualitative Analysis of Biofilm Formation

B. cereus ATCC 14579 and the corresponding $\Delta sRNA^{other}$ strain were assayed for biofilm formation at the liquid-air interface. Strains were grown to stationary phase (18 h) in 5 mL of LB at 37 ^{o}C with shaking. Overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.1 in fresh LB containing 500 μM 2',2'-dipyridal (dip). Aliquots of 20 ml of diluted fresh culture were added to 25 ml conical vials, and glass microscope slides were inserted into the vials to create a liquid air interface. Both the wt and deletion strains were incubated at 30 ^{o}C and 37 ^{o}C at an angle of 45^{o} for 24 and 48 h.

The extent of biofilm formation was determined by microscopic analysis. After the desired time, the microscope slides were removed from the vials. The bottom side of

the slide was wiped using a Kimwipe and the top of the slide was rinsed with 5-7 mL of LB. Each microscope slide was stained with 3-4 drops of safranin (2.5%) for 15 min and rinsed with distilled water. Once again, the bottom of the slide was wiped using a Kimwipe containing ethanol to remove any excess safranin that may have seeped on to the back of the slide. After air drying, biofilm formation was examined using phase contrast microscopy (400x magnification) (Axio Observer D1, Zeiss).

Quantitative Analysis of Biofilm Formation

Three different 96-well microtiter plates were tested for noticeable biofilm formation: IMMUNOLN®2HB (NT520913), Falcon Microtest Flat Bottom (Becton Dickinson 351172), and Biolog MircoPlate Trays (30311). Noticeable biofilm formation developed only on the IMMUNOLN®2HB after 48 h at 37 ^{o}C. Therefore, *B. cereus* ATCC 14579 and Δs*RNA*^{other} strains were assayed for biofilm formation by the use of this 96-well microtiter plate and Luria-Bertani (LB) medium containing 500 μM dip. Cultures of both strains were prepared as stated above. Once cultures were diluted to an OD_{600} of 0.1 in fresh LB with or without 500 μM dip, aliquots of 200μl of diluted fresh culture were added to each well in the 96-well microtiter plate and incubated at 37 ^{o}C for 48 hr.

Biofilm formation was quantified using the microtiter plate biofilm assay as published in *Current Protocols in Microbiology* (12) with the following exceptions. Wells were washed by squirting PBS from a wash bottle into each individual well. Biofilms were stained using 3 drops of crystal violet (1 %) for 20 min and solubilized using 250 μl of 95 % ethanol. Absorbance was measured at a wavelength of 590 nm.

Results

In the current study, differences in biofilm formation between *Bacillus cereus* ATCC 14579 and the corresponding ΔsRNA^{other} strain were assessed at the liquid-air interface. Qualitative analysis showed that biofilms formed at the liquid-air interface on glass slides only with addition of the iron chelator, dip. Under microscopic analysis, wt *B. cereus* was shown to grow more dense biofilms than the deletion strain. However, a difference in the structure of the biofilms can be noted. While the wt strain grows thick uniform biofilms, the sRNA^{other} deletion strain forms biofilms that have a "swirly", monolayer appearance. It appears that the wt produces biofilms with a high cell density, whereas the sRNA^{other} deletion strain produces biofilms with a lower cell density. Cell density differences were noted by the overlapping structure of the wt biofilm and the monolayer biofilm of the corresponding ΔsRNA^{other} strain.

While a noticeable difference in biofilm formation between the wt and the corresponding ΔsRNA^{other} strain occurred after 24 h at 37 ^{o}C, biofilms grown for 24 h and 48 h at 30 ^{o}C showed similar appearances for both the wt and ΔsRNA^{other} strains. Whereas wt and ΔsRNA^{other} strains formed biofilms that began to disperse after 48 h at 37 ^{o}C, there was no apparent disassociation of either biofilm after 24 h or 48 h at 30 ^{o}C (Fig 1).

Qualitative analysis indicated a noticeable difference in biofilm formation; therefore, quantitative analysis was conducted to better understand the differences in biofilm formation between the wt and corresponding ΔsRNA^{other} strain. Quantitative analysis of biofilm formation at the liquid-air interface of the plastic 96-well microtiter plate showed no significant difference in biofilm formation with or without the addition

of dip. However, a significant difference in absorbance can be noted in biofilm formation between the wt and $sRNA^{other}$ deletion strains (Fig 2). The wt demonstrated a 23 ± 2.1 % higher absorbance than the deletion strain when grown in LB alone. When grown in LB + dip, the wt had a 14 ± 4.2 % higher absorbance than the corresponding $\Delta sRNA^{other}$ strain. Absorbance measurements were taken at 590 nm and data was presented as normalized to wt *B. cereus* grown in LB.

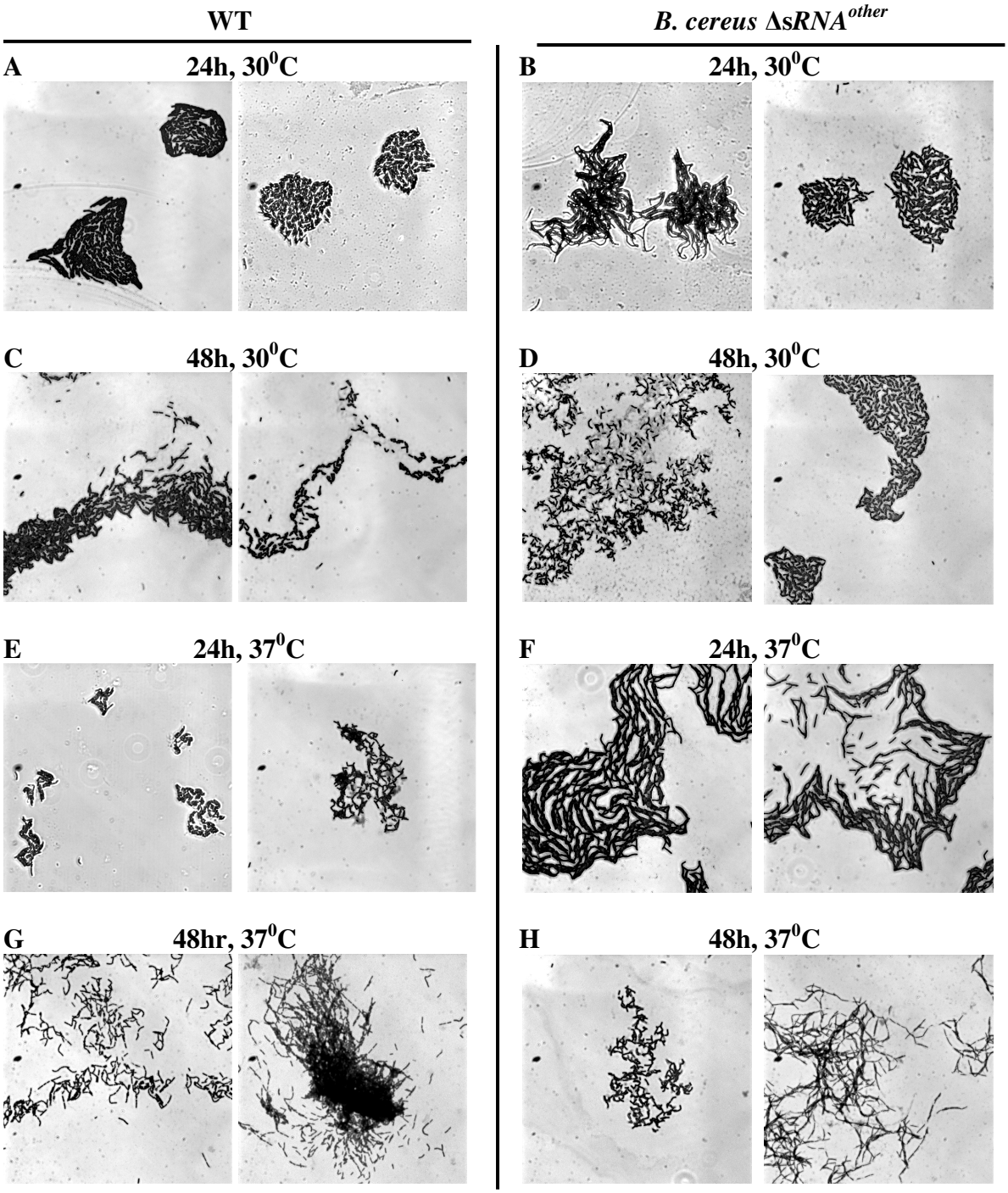

Figure 1. Biofilm formation in wt and *B. cereus* Δs*RNA*^{other}. WT *B. cereus* (A, C, E, and F) and *B. cereus* Δs*RNA*^{other} (B, D, F, and G) biofilms were formed on glass slides at 30 °C (A-D) or 37 °C (E-H) for 24 hours (A, B, E, and F) and 48 hours (C, D, G, and H). The two pictures per strain and condition are representative of the observed biofilm structures.

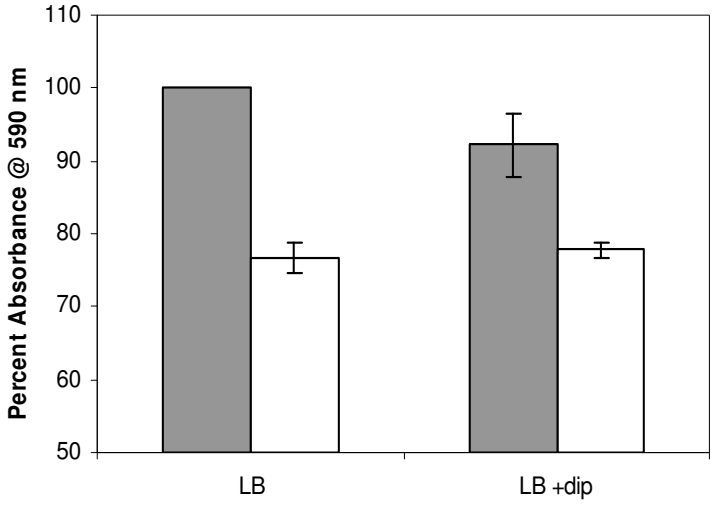

Figure 2. Biofilm formation of wt (grey bars) and *B. cereus* Δ*sRNA*^{Other} (white bars) grown under high and low iron conditions. Data is presented as normalized to wt *B. cereus* grown in LB. Y-error bars represent standard deviation among mean values for three individual trials.

Discussion

Our investigations qualitatively and quantitatively demonstrate the structural differences and density variations in biofilm formation between *B. cereus* ATCC 14579 and *B. cereus* Δs*RNA*^{other}. Current studies demonstrate differences in cell wall morphology and antibiotic resistance between *B. cereus* ATCC 14579 and the corresponding deletion strain, *B. cereus* Δs*RNA*^{other} (10).

Recent evidence has shown *B. cereus* biofilms to be more resistant to cleaning procedures and antibiotic treatments than planktonic cells (2), therefore the ability of *B. cereus* to form biofilms may lead to problems in the food production or water treatment industries (1, 8). Previous studies have shown that *B. cereus* preferentially forms biofilms at the liquid-air interface (2). These findings indicate that biofilm formation primarily develops in industrial storage and piping systems that are partially filled during operation or areas where residual liquid remains after the production cycle (2). Other factors

influencing biofilm formation include; strain, incubation time and environmental conditions, such as the composition of the medium and surface of adherence (2).

Iron availability in the environment may also play an important role in biofilm formation. The role of iron availability in biofilm formation varies among microorganisms. For example, iron availability induces biofilm formation in *Staphylococcus aureus*, while it inhibits biofilm formation in other species such as *Vibrio cholerae* (3). The amount of iron availability *in vitro* was altered using chemical compounds such as the iron chelator dip. Adding dip to the media reduced the amount of iron present and increased the expression of $sRNA^{other}$, whereas with high iron conditions, expression of $sRNA^{other}$ was repressed. Biofilm formation was seen only with dip addition when cells were grown on glass slides. However, biofilm formation occurred on the plastic 96-well plate with and without the addition of dip. This difference may be due to the surface characteristics of the colonized area, such as roughness and composition of the material (7).

Qualitative analysis showed structural differences in biofilm formation between the wt and deletion strains. These structural differences may be caused by differences in cell differentiation. Extensive cellular differentiation can occur within the extracellular matrix (8). Based on mutant phenotypes from the organisms; *Vibrio cholerae*, *Escherichia coli*, *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* biofilm formation can be divided into five genetically distinct stages: initial surface attachment, monolayer formation, migration to form multilayered microcolonies, production of extracellular matrix and biofilm maturation with characteristic three-dimensional architecture (8). Applying these five genetically distinct stages to our investigations of

biofilm formation helps to better understand the structural differences in biofilm formation between the wt and *B. cereus* Δs*RNA*^{other} strains. The deletion strain appears to only develop a monolayer biofilm on a glass surface within 48 h, whereas the wt strain appears very concentrated in some areas as a result of multiple biofilm layers. The ability of the wt strain to form more dense biofilms than the corresponding deletion strain may cause variations in antibiotic resistance and may be related to differences in cell wall morphology between the wt and deletion strains.

In conclusion, the deletion of sRNA^{other} negatively affects *B. cereus* biofilm formation resulting in a monolayer biofilm, yet its specific role is not understood. Deletion of sRNA^{other} also results in changes in cell wall morphology and reduced resistance to numerous unrelated antibiotics. It can be hypothesized that sRNA^{other} plays a role in cellular development. Further investigations may include scanning electron microscopy in order to gain a better understanding of the structural differences of the biofilm. Protein and carbohydrate analysis of the extracellular matrix could also be advantageous to understanding the structural differences between the wt and deletion strains. Other surfaces could also be tested for biofilm formation. From an environmental viewpoint, materials used in water treatment plants, such as plastic or metal devices used to transport or store water could be tested for biofilm formation. Continuing investigations may help us develop new cleaning procedures or antibiotic treatments to help reduce and battle biofilm formation.

References

1. **Auger, S., K. Evelyne, S. Aymerich, and M. Gohar.** 2005. Autoinducer 2 Affects of Biofilm Formation by *Bacillus cereus*. Appl Environ Microbiol. **72**:937-940.

2. **Wijman, J., P. de Leeuw, R. Moezelaar, M. Zwietering, and T. Abee.** 2007. Air-Liquid Interface Biofilms of *Bacillus cereus*: Formation, Sporulation, and Dispersion. Appl Environ Microbiol. **73**:1481-1487

3. **Wu, Yun., F. Wayne Outten.** 2009. IscR Controls Iron-Dependent Biofilm Formation in *Escherichia coli* by Regulating Type I Fimbria Expression. J Bacteriol. **191**:1248-1257

4. **Wiley, J., L. Sherwood, and C. Woolverton.** Prescott, Harley, and Kline Principles of Microbiology 7th ed. McGraw Hill. 2008. 143-4.

5. **Stewart, P., J. Costerton.** 2005. Antibiotic Resistance of Bacteria in Biofilms. Lancet. **358**:135-138

6. **Anderl, J., M. Franklin, and P. Stewart.** 2000. Role of Antibiotic Penetration Limitation in *Klebsiella pneumoniae* Biofilm Resistance to Ampicillin and Ciprofloxacin. Antimicrob Agents Chemother. **44**:1818-1824

7. **Tsvetanova, Zvezdimira.** 2006. Study of Biofilm Formation on Different Pipe Materials in a Model of Drinking Water Distribution System and its Impact on Microbial Water Quality. Springer. 463-468

8. **Lemon, K., A. Earl, H. Vlamakis, C. Aguilar, and R. Kolter.** 2008. Biofilm Development with an Emphasis on *Bacillus subtilis*. Curr Top Microbiol Immunol. **322**:1-16

9. **Ataide, S., T. Rogers, and M. Ibba**. 2009. The CCA anticodon specifies separate functions inside and outside translation in *Bacillus cereus*. RNA Bio. **6:4**, 479-87

10. **Rogers, T.** 2010. Elucidating the Function of a Pseudo-tRNA in Bacillus cereus. Dissertation. The Ohio State University, Columbus, OH.

11. **Mey, A. R., S. A. Craig, and S. M. Payne.** 2005. Characterization of Vibrio cholerae RyhB: the RyhB regulon and role of ryhB in biofilm formation. Infect Immun **73**:5706-19.

12. **Merritt, J., D. Kadouri, and G. O'Toole**. *Current Protocols in Microbiology* (2005) 1B.1.1-1B.1.3. 2005. John Wiley & Sons.

13. **Ataide, S., B. Jester, K. Devine, and M. Ibba**. 2005. Stationary-phase expression and aminoacylation of a transfer-RNA-like small RNA. EMBO **6:8**,742-6.